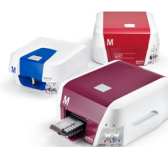




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Anti-Human CD73 Monoclonal Antibody Inhibits Metastasis Formation in Human Breast Cancer by Inducing Clustering and Internalization of CD73 Expressed on the Surface of Cancer Cells

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Recent studies have shown that Abs that target the cell-surface enzyme CD73 (ecto-5'-nucleotidase) reduce growth of primary tumors and metastasis in syngenic mice by inhibiting the catalytic activity of CD73, and thus increasing the activity of cytotoxic T lymphocytes. In this article, we report another anticancer mechanism of anti-CD73 Abs and show that an anti-CD73 mAb (AD2) inhibits metastasis formation by a mechanism independent of CD73 catalytic activity and inhibition of primary tumor growth. This mechanism involves clustering and internalization of CD73, but does not require cross-linking of CD73, because both whole IgG anti-CD73 AD2 mAb and Fab' fragments thereof exhibited this effect. Ex vivo treatment of different breast cancer cell lines with anti-CD73 AD2 mAb before i.v. injection into mice inhibited extravasation/colonization of circulating tumor cells and significantly reduced metastasis development. This effect was also observed when the cancer cell-surface expression of CD73 was significantly reduced by small interfering RNA knockdown. The antimetastatic activity is epitope specific, as another Ab that efficiently binds CD73-expressing live cancer cells did not lead to CD73 internalization and metastasis inhibition. Furthermore, anti-CD73 AD2 mAb inhibited development of metastasis in a spontaneous animal model of human metastatic breast cancer. Our study shows that some anti-CD73 mAbs cause cell-surface clustering of CD73 followed by internalization, thus inhibiting the ability of circulating tumor cells to extravasate and colonize, leading to inhibition of metastasis. Ab-based CD73 cancer therapy should include a combination of Abs that target the catalytic activity of CD73, as well as those with the characteristics described in this article. *The Journal of Immunology*, 2013, 191: 4165–4173.

Metastasis is a complex process that involves a series of events, including local invasion, intravasation, survival in the circulation, extravasation, and ultimately colonization of cancer cells in a distant organ. Many proteins have been shown to be involved in the metastatic process, including tumor cell plasma membrane proteins that may cross talk with the surrounding environment, such as the extracellular matrix and adjacent cells (reviewed in Ref. 1). One of these metastasis-associated

membrane proteins is CD73 (ecto-5'-nucleotidase), a 70-kDa GPI-anchored protein. In a global proteomic analysis of isogenic metastatic and nonmetastatic breast cancer cell lines, we showed that CD73 was more highly expressed in metastasizing versus nonmetastasizing cancer cells, and analysis of clinical breast cancer biopsies demonstrated a correlation between CD73 expression in primary tumors and risk for distant metastasis (2, 3).

CD73 is ubiquitously expressed in most normal tissues, including endothelial and subsets of hematopoietic cells (4); however, increased levels are present in a number of cancers (5). CD73 has been proposed to have several functions, including cell adhesion and enzymatic conversion of AMP to adenosine and inorganic phosphate (6–10). It was recently shown in a murine syngenic breast cancer model that inhibition of adenosine production using anti-murine CD73 mAb or α , β -methylene adenosine-5'-diphosphate (APCP) decreased tumor growth. Adenosine is known to inhibit the effect of CD8⁺ T cell-mediated response; thus, the mechanism behind the tumor growth inhibition was induction of such CD8⁺ T cell-mediated antitumor responses in the absence of adenosine (5, 8). Inhibiting adenosine production also inhibited metastasis development by decreasing migration of cancer cells (8). In addition, studies in CD73 knockout mice showed that lack of host-derived CD73 significantly inhibited both tumor growth and metastasis formation, indicating that not only CD73 expressed on cancer cells, but also CD73 expressed by adjacent normal tissues plays a critical role in the cancer process (7, 11). Treatment of cancer cells with APCP or RNA interference silencing of CD73 decreased adhesion to extracellular matrix proteins, laminin and fibronectin (12). Interestingly, CD73 RNA interference was shown to inhibit adhesion and invasion

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The online version of this article contains supplemental material.

Abbreviations used in this article: APCP, α , β -methylene adenosine-5'-diphosphate; MFI, mean fluorescence intensity; ROI, region of interest; siRNA, small interfering RNA.

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of cancer cells more potently than APCP, suggesting that the capacity of CD73 to promote adhesion and invasion may relate to other mechanisms in addition to its catalytic activity (12).

Although most studies have analyzed murine CD73 in syngenic mouse models, in this study, we examined the effect of anti-human CD73 Abs in a human breast cancer xenograft model. Using advanced live animal bioimaging, we identified an anti-human CD73 Ab that significantly decreased spontaneous metastasis formation independent of CD73 catalytic activity. The anti-CD73 mAb (AD2) that exhibits this novel mechanism of action alters the normal distribution of CD73 from being evenly expressed in the membrane to forming clusters followed by internalization. Blocking cell-surface-expressed CD73 reduced the ability of cancer cells to extravasate from the circulation and colonize at distant sites.

Materials and Methods

Cell lines, culture, and transfection

The MDA-MB-231 and M4A4 LM3-2 cell lines (American Type Culture Collection) were transfected with luciferase 2 (LUC2)-expressing lentiviral particles (In Vivo Imaging Solutions) using a multiplicity of infection virus particle concentration of 15 according to the manufacturer's guidelines, generating stably luciferase-expressing MDA-MB-231 LUC2 (MDA-MB-231) and M4A4 LM3-2 LUC2 (LM3) cell lines. The two cell lines were maintained in DMEM (Life Technologies, Invitrogen) supplemented with 10% FBS (Life Technologies), 5% penicillin/streptomycin (Life Technologies). Cultures were grown in humidified atmosphere of 5% CO₂ at 37°C. All cell lines were routinely checked for *Mycoplasma* infection and morphology changes, and propagated no longer than 3 mo after resuscitation.

Abs

The mouse anti-human CD73 AD2 mAb (a gift from Prof. L. Thompson, Oklahoma Medical Research Foundation, Oklahoma City, OK) was produced at the Immunology Core Facility at the University of North Carolina, Chapel Hill. The mouse anti-human CD73 IE9 mAb was purchased from Santa Cruz Biotechnologies. Both mAbs were generated by immunization with full-length CD73 purified from human placenta (4). The isotype-matched control mAb was an IgG1κ α-Diphtheria toxoid mAb (HYP 123-05; Bioporto). Alexa Fluor 555 labeling was performed using Alexa Fluor 555 mAb Labeling Kit according to the manufacturer's guidelines (Invitrogen). The anti-C3d Ab (AF2655) used for immunohistochemical analysis was purchased from R&D Systems.

Generation of Fab' fragments of anti-CD73 AD2 mAbs

To generate Fab' fragments, we initially dialyzed anti-CD73 AD2 mAb against 100 mM acetate buffer, 1 mM EDTA, pH 4.5, and incubated them for 72 h with pepsin (5 μg/mg Ab; Sigma-Aldrich). The generated F(ab')₂ fragments were separated by size exclusion chromatography using 100 mM acetate buffer, 1 mM EDTA, and 50 mM cysteine pH 5.5 on a Superdex 200 HiLoad 16/60 column (GE Healthcare) and incubated for 24 h with papain (10 μg/mg Ab; Sigma-Aldrich). The generated Fab' fragments were separated by size exclusion chromatography using PBS, pH 7.4 on a Superdex 200 HiLoad 16/60 column (GE Healthcare).

CD73 knockdown

Human CD73 small interfering RNA (siRNA; #S9735 and #S9436; Life Technologies) and scrambled control siRNA (Qiagen, Denmark) was transfected into MDA-MB-231 LUC2 cells either in combination or as single siRNAs (300 nM) in 100 μl nucleofector solution V (Cell Line Nucleofector Kit V; Amaxa) using an Amaxa Nucleofector II according to the manufacturer's guidelines (Lonza Europe). Knockdown was evaluated using flow cytometry as described later. Injection of the cancer cells in the tail vein was performed 96 h after transfection.

In vivo studies of anti-CD73 AD2 mAb treatment in a spontaneous metastasis xenograft model

Subconfluent cells in culture were washed in PBS and harvested by a cell scraper (Almeco). Cells (1 × 10⁶) were resuspended in a 1:1 mixture of extracellular matrix from the Engelbreth-Holm-Swarm sarcoma (Sigma-Aldrich) and DMEM before inoculation. Orthotopic transplantation of cancer cells was performed by injection into a surgically exposed mammary gland of anesthetized 8-wk-old female CB-17 SCID mice (Taconic). Anti-CD73

AD2 mAb (5 mg/kg) and control mAb (5 mg/kg) were injected i.p. 4 d after cancer cell transplantation into groups of five animals and subsequently biweekly for 5 wk. Primary tumors were removed from anesthetized mice using blunt instrument dissection. All animal experiments were approved by The Experimental Animal Committee, The Danish Ministry of Justice and were performed at the animal facility core at University of Southern Denmark. The mice were housed under specific pathogen-free conditions with ad libitum food and drinking water. The mice were euthanized if they showed any adverse signs or symptoms of disease including weight loss, paralysis, or general discomfort.

Ex vivo pretreatment of cancer cells with anti-human CD73 mAb

Cells were harvested by scraping, then were washed and incubated with anti-CD73 AD2 mAb (10 μg/ml), anti-CD73 IE9 mAb (10 μg/ml), or control mAb (10 μg/ml) at 37°C for 2 h. For flow cytometry analysis of CD73 expression, the cells were washed three times in ice-cold PBS and subsequently stained with Alexa Fluor 647 goat anti-mouse Ab at 4°C and incubated with a second round of primary Ab at 4°C for 30 min before staining with Alexa Fluor 647 goat anti-mouse Ab. Analysis of the re-expression of CD73 was performed by AD2 incubation at 37°C followed by washing three times and subsequent 3-h incubation in complete medium without AD2 at 37°C. Thereafter, the cells were washed three times and then incubated with a second round of AD2 Ab at 4°C for 30 min before Alexa Fluor 647 goat anti-mouse Ab staining.

Data acquisition and analysis was performed as described in the *Flow Cytometry* section.

Before tail vein injections, the cells were resuspended in PBS at a concentration of 5 × 10⁶/ml and 0.2 ml was injected into the tail vein of 6- to 8-wk-old female CB-17 SCID mice.

In vivo imaging

Relative quantification of tumor burden and metastasis development was performed weekly using whole-body bioluminescent imaging (IVIS Spectrum; Caliper Life Science). Mice were injected with D-luciferin (150 mg/kg body weight) and then anesthetized with isoflurane gas. Images were acquired starting 10 min after luciferin injection. Regions of interest (ROI) were drawn around the mammary fat pad tumor. Further, ROIs were drawn encircling each of the two lungs to quantify metastases. The photon emission transmitted from the ROIs was quantified in photons/s/cm²/sr using Caliper Life Science Living image (version 4.0). The animals were euthanized 10 min after luciferin injection to image excised organs, and organs were imaged 2 min later.

Flow cytometry

Subconfluent cells in culture were washed in PBS, harvested by scraping, and washed (300 × g, 5 min, 4°C) in DMEM supplemented with Complete Mini Protease Inhibitor mixture (complete DMEM; Roche Diagnostics). PBMCs were isolated using standard Ficoll-Paque according to manufacturer's protocol (GE Healthcare). Cells (1 × 10⁵) were incubated with 10 μg/ml anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb for 30 min at 4°C, washed twice in complete DMEM, and incubated with an Alexa Fluor 647 goat anti-mouse Ab (Invitrogen) for 60 min at 4°C. The cells were then washed, resuspended in PBS, and analyzed on a FACS-Calibur (Becton Dickinson). The collected data were analyzed using FlowJo software (Tree Star).

Inhibition of catalytic activity

Cells (5 × 10⁴) were resuspended in 100 μl glycine buffer (75 mM glycine, 5 mM MgSO₄, pH 7.4) containing various concentrations of anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, control mAb, or 0.1 mM APCP (Sigma Aldrich). Cells treated with Ab or APCP were analyzed with and without 0.2 mM AMP and incubated for 3 h at 37°C. When analyzing the catalytic activity of cancer cells in the setup resembling the tail vein injection experiments, cancer cells were incubated with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb for 2 h, washed three times in TBS, resuspended in glycine buffer with and without 0.2 mM AMP, and incubated for 1 h at 37°C. After incubation with AMP, 50 μl of 0.5 M H₂SO₄ was added to each well, followed by 50 μl phosphate reaction solution (0.4% NH₄-molybdate, 10% ascorbic acid), and incubated for 30 min at room temperature while shaking to measure the generated inorganic phosphate, that is, the conversion of AMP to adenosine. The color-reaction product (molybdenum blue) was colorimetrically measured at 560 nm using a Victor3 Multilabel Plate Reader (PerkinElmer Life Sciences). The catalytic activity was determined by subtracting the values in the absence versus the presence of AMP.

Anchor-independent growth

Soft agar assay was performed as previously described by Ke et al. (13) with minor modifications. In brief, a prelayer of 25 μ l prewarmed 2 \times IMDM (Invitrogen) and 25 μ l 1.2% agar (Sigma) was plated into 96-well microplates. MDA-MB-231 cells (5×10^3) were seeded on top of the prelayer in 20 μ l 2 \times IMDM and 30 μ l 0.8% agar containing anti-CD73 AD2 mAb or 10 μ g/ml control mAb. Finally, a semisolid feeder top layer of 25 μ l 2 \times IMDM and 25 μ l 0.2% agar were seeded on top of the cell-containing layer. The cells were incubated in a humidified atmosphere of 5% CO₂ and 37°C for 12 d. Anchored independent growth was quantified by bioluminescence using a Victor 3 Multilabel Plate Reader.

Cell migration assay

Migration assays were performed as previously described with minor modifications (14). After MDA-MB-231 cells (5×10^5 cells) were seeded in six-well plates, a scratch was performed using a pipette tip. After washing for four times in PBS, 3 ml serum-free DMEM containing 10 μ g/ml anti-CD73 AD2 mAb or control mAb was added. Snapshots of migrating cells were taken after 3 and 48 h using a Nikon Coolpix P5100 camera mounted on a Leica DMIL microscope and converted into segmented dark-field images in Photoshop. The comparison of migration after Ab treatment was performed by quantifying the segmented dark-field images using ImageJ. Each scratch was normalized to the quantification of the starting point 3 h postscratching.

Immunofluorescence microscopy

Cells (1×10^5) were seeded in 12-well plates on coverslips and, 24 h postseeding, incubated with anti-CD73 AD2 mAb (IgG or Fab', 10 μ g/ml) or anti-CD73 IE9 mAb (10 μ g/ml) for 2 h at 37°C. After incubation, the cells were counterstained for 10 min with 5 μ g/ml Alexa 633-conjugated wheat germ agglutinin (Invitrogen), washed in PBS, and fixed for 15 min with 4% formaldehyde. After permeabilization using 0.05% Triton-X 100 (Bio-Rad Laboratories), the cells were blocked with 1% BSA (Sigma-Aldrich) before staining with Alexa Fluor 488-conjugated anti-mouse Ab (Invitrogen) for 30 min at 4°C. The coverslips were mounted on glass slides in antifade mounting medium (Invitrogen). Imaging was performed with an Olympus FV1000 confocal laser-scanning microscope using laser lines 405, 488, and 635 for excitation, appropriate emission filters, and 20 \times NA:0.95 water immersion objective.

Live cell fluorescence microscopy

Cells (1×10^5) were seeded in 12-well plates on coverslips. After 24 h, the cells were washed, incubated with Alexa Fluor 555-conjugated anti-CD73 AD2 mAb (10 μ g/ml) for 10 min and washed again before monitoring with spinning disc microscopy performed with a Nikon Eclipse Ti (Nikon Industries) microscope equipped with a Yokogawa CSU-X1 spinning disc unit (Yokogawa Electric Corporation), Andor Laser launcher, and an Andor EMCCD (Andor Technology) for detection. Imaging was performed using the 561 laser line for excitation and a 593/40 emission filter. The integration time was 40 ms, and a 60 \times oil objective was used.

Immunohistochemistry

Generation of tissue arrays of cell line-derived tumors and staining procedures were performed as previously described (2, 15).

Proliferation assay

Cells were incubated with anti-CD73 AD2 mAb (0.01–10 μ g/ml) or control mAb (0.01–10 μ g/ml) at 37°C for 2 h. Cells (5×10^3) were seeded in 96-well plates in quintuplicate. After 96 h, proliferation was measured in an MTT proliferation assay (Invitrogen) according to the manufacturer's guidelines.

Statistics

The statistical significance of bioluminescence measurements of tumor growth or metastasis in different groups was calculated using Student *t* test or random-effect models when appropriate. The *p* values >0.05 were considered nonsignificant.

Results

Anti-human CD73 mAb binds human, but not mouse, CD73 and detects human xenograft tumors in mice

Flow cytometry analysis showed that anti-CD73 AD2 mAb (AD2 mAb) and anti-CD73 IE9 mAb (IE9 mAb) both recognized CD73 on the human breast cancer cell lines LM3 and MDA-MB-231, but

failed to detect the murine CD73 variant expressed on isolated mouse mononuclear hematopoietic cells (Fig. 1A). Furthermore, when cells were costained with AD2 and IE9 mAbs, the saturation signals increased compared with staining with each Ab alone, indicating that AD2 and IE9 recognize different epitopes on CD73 (Supplemental Fig. 1A). The expression of CD73 in LM3 is ~20% of that in MDA-MB-231 cells.

The specificity of AD2 mAb toward human CD73 was further tested by injecting Alexa Fluor 647-labeled AD2 mAb or control mAb into mice with xenograft tumors established from LM3 breast cancer cells. In vivo fluorescence bioimaging showed that only AD2 mAb detected the primary tumor, and that the AD2 mAb remained visible above background after 72 h (Fig. 1B). No accumulation of AD2 mAb was observed in any mouse organs, demonstrating that the AD2 mAb only recognizes human CD73, and not murine host CD73.

Anti-human CD73 mAb inhibits establishment of spontaneous metastasis, but not primary tumor growth

Next, we examined the ability of AD2 mAb to inhibit primary tumor growth in vivo. LM3 breast cancer cells were inoculated into the mammary fat pad of immunodeficient mice. Starting from day 4 after cancer cell implantation, mice received AD2 mAb (*n* = 5, 5 mg/kg) or control mAb (*n* = 5, 5 mg/kg) i.p. twice a week for 5 wk, whereas the primary tumor growth was simultaneously quantified using bioluminescence imaging. No difference in growth rate of

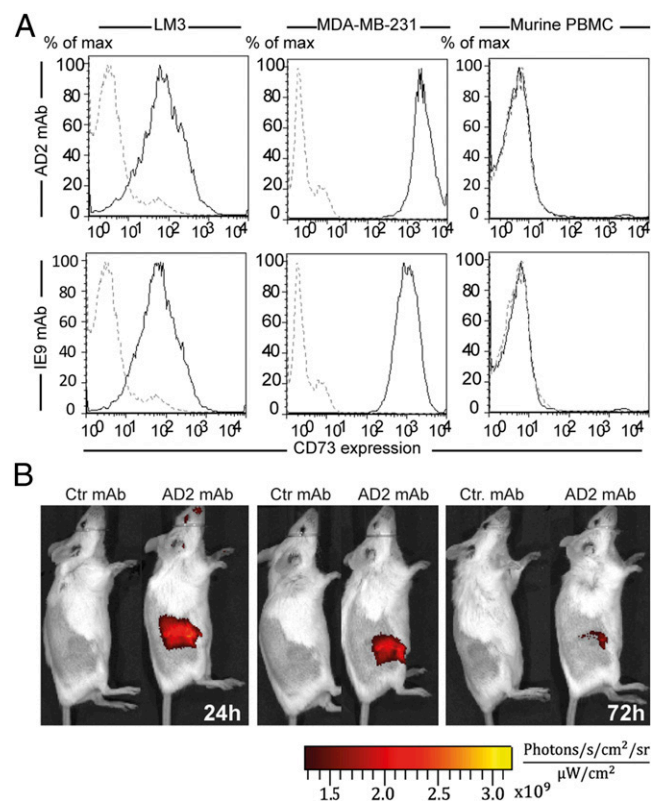


FIGURE 1. The anti-human CD73 mAb binds human, but not murine, CD73 and persists within tumors for at least 72 h after treatment. (A) Flow cytometry analysis showing binding of the anti-CD73 AD2 mAb and anti-CD73 IE9 mAb to human MDA-MB-231 and LM3 cells, but not to murine mononuclear cells (black solid line). No binding of control mAb (gray dashed line) was observed for MDA-MB-231, LM3, or murine PBMCs. (B) Alexa Fluor 647-labeled anti-human CD73 mAb or control mAb were injected i.p. in LM3 tumor-bearing mice. Fluorescence corresponding to the level of mAb binding to the primary tumor was measured after 24, 48, and 72 h using an IVIS Spectrum instrument.

the tumors was observed between the two groups when the relative light emission was compared, indicating that AD2 mAb does not inhibit primary tumor growth (Supplemental Fig. 1B, 1C).

Furthermore, MTT proliferation assays showed that AD2 mAb had no effect on the proliferation of cancer cells in vitro (Supplemental Fig. 1D, 1E), demonstrating that the AD2 mAb is neither cytostatic nor cytotoxic, supporting the in vivo observations.

We next examined whether AD2 mAb had an effect on the ability of cancer cells to establish spontaneous metastasis in the lungs. The primary tumors were surgically removed 5 wk after LM3 cancer cell inoculation when they reached a size of 1.2 cm in diameter, and the metastatic burden in the lungs was quantified weekly for 5 wk using bioluminescence (Fig. 2A). At end point, 10 wk after LM3 cancer cell inoculation, the overall metastatic burden was 20 times lower in the mice treated with AD2 mAb ($n = 5$) than those treated with control mAb ($n = 5$; $p < 0.05$; Fig. 2B), demonstrating that the AD2 mAb inhibits spontaneous metastasis formation. Because the complement system is still active in CB-17 SCID mice and may be involved in the elimination of cancer cells, we examined the complement activation factor C3d deposits in the primary tumors from mice treated with AD2 mAb and control mAb by immunohistochemistry and found no indication of complement activation by the AD2 mAb or control mAb (Supplemental Fig. 1F, 1G).

Because migration of cancer cells is central in the metastatic process, we investigated whether AD2 mAb inhibited the migratory potential of cancer cells in vitro. However, comparing the migration of cancer cells in a wound-healing scratch assay showed no difference between cancer cells treated with AD2 mAb compared with those treated with control mAb (Supplemental Fig. 2A).

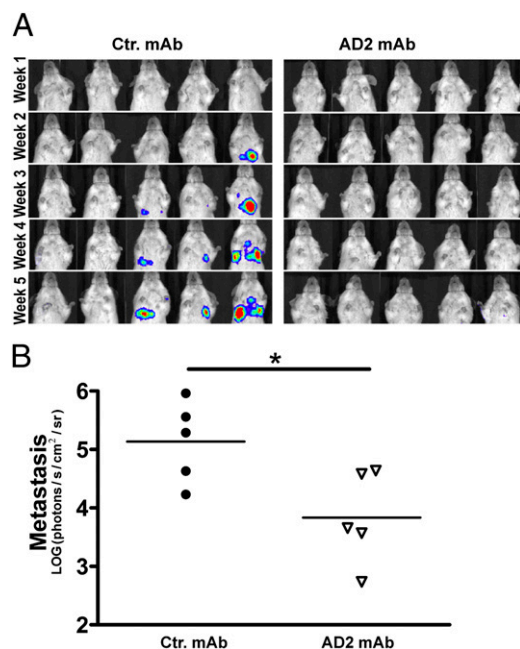


FIGURE 2. Anti-CD73 AD2 mAb inhibits metastasis in a spontaneous metastasis model. LM3 cells (10^6) were transplanted into the mammary fat pad of SCID mice, and anti-CD73 AD2 mAb (5 mg/kg) or control mAb (5 mg/kg) were administered at day 4 and subsequently twice a week for 5 wk. (A) After surgical removal of the primary tumors, the animals were imaged weekly for potential lung metastasis by measuring luciferase activity in the lungs using an IVIS Spectrum instrument. (B) Nonspecific reflection of light on paws was graphically removed. End-point measurements (5 wk after primary tumor removal) of photon radiance per area were compared between the two groups of five animals each using the Student t test ($*p < 0.05$).

Ex vivo preincubation of LM3 cells with anti-CD73 AD2 mAb inhibits colonization, extravasation, or both

To further study how the anti-human CD73 mAbs inhibit metastasis development, we pretreated LM3 (10^6) cells with 10 μ g/ml AD2 mAb or control mAb ex vivo and injected the cells into the tail vein of immunodeficient mice ($n = 6$ in each group) to study the process of extravasation, colonization, or both. Ex vivo incubation of LM3 cells with AD2 mAb significantly inhibited metastasis formation when compared with LM3 cells incubated with control mAb ($p < 0.05$; Fig. 3A). Furthermore, no visible metastasis was detected (0/6) in lungs from mice injected with AD2 mAb-treated LM3 cells examined ex vivo by bioluminescence, whereas metastases were present in five of six mice injected with control mAb-treated LM3 cells. Furthermore, ex vivo examination of excised rib cages from mice injected with LM3 cancer cells revealed that bone metastases were present in 100% (3/3) of mice treated with control mAb, but in none of those treated with AD2 mAb. This demonstrates that AD2 mAb inhibits bone and lung metastasis. It should be noted that bone metastases were present in animals with no detectable lung metastases, suggesting that the bone metastases were not secondary to lung metastases (Fig. 3B).

Ex vivo preincubation of MDA-MB-231 with anti-CD73 AD2 mAb, but not anti-CD73 IE9 mAb, inhibits metastasis formation in mice

To further study the anti-human CD73 mAb effect on metastasis development, we pretreated another human metastatic breast cancer cell line, MDA-MB-231 (10^6) cells with 10 μ g/ml AD2 mAb, IE9 mAb, or IgG control mAb ex vivo and injected the cells into the tail vein of immunodeficient mice ($n = 6$ in each group). AD2 mAb

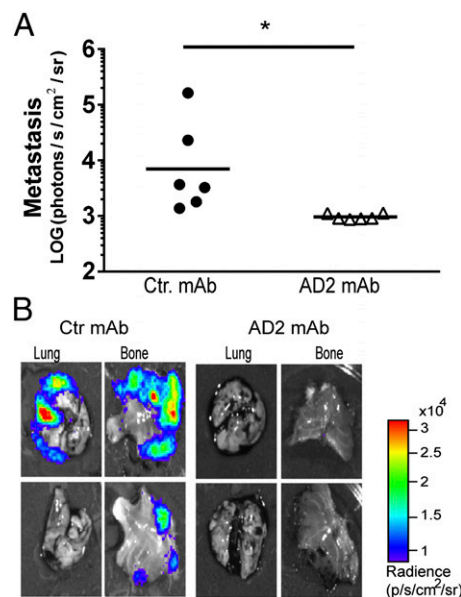


FIGURE 3. Ex vivo incubation of LM3 cells with anti-CD73 AD2 mAb inhibits metastasis formation after i.v. injection. LM3 cells (1×10^6) were incubated ex vivo with anti-CD73 AD2 mAb or control mAb for 2 h and subsequently injected into the tail vein of female CB-17 SCID mice. (A) Lung metastasis development in mice, injected with LM3 cells incubated ex vivo with either anti-CD73 AD2 mAb or control mAb ($n = 6$ per group), was monitored by bioluminescence imaging (photon radiance per area from the lung region), and the results at week 16 were compared using Student t test ($*p < 0.05$). (B) Metastasis development in four representative excised lungs and rib cages (week 16) from mice injected with LM3 cells incubated ex vivo with either anti-CD73 AD2 mAb or control mAb, as determined by bioluminescence imaging (excised lungs and corresponding rib cages from the same animal are depicted in the same row).

preincubation significantly reduced MDA-MB-231–derived lung metastasis formation compared with those preincubated with control mAb ($p < 0.05$; Fig. 4A, 4B). In contrast, IE9 mAb did not inhibit metastasis formation of MDA-MB-231 compared with control mAb-treated cells (Fig. 4D, 4E).

Immunohistochemical staining with an anti-pan-cytokeratin Ab of lungs from mice injected with MDA-MB-231 cells treated with either AD2 mAb or control mAb demonstrated a significantly lower number of metastatic foci in the lungs of mice injected with MDA-MB-231 cells treated with AD2 mAb compared with those injected with MDA-MB-231 cells treated with control mAb (Fig. 4C; $p < 0.001$). Furthermore, the size of the metastases was generally smaller in the AD2 mAb-treated versus control mAb-treated group, in agreement with the bioimaging results. The metastasis was located in the alveolar parenchyma, both in the alveolar wall and in the alveolar lumen, and did not differ between the two treated

groups. No stromal reaction was observed, and the overall structure of the lung was normal.

Next, we reduced the MDA-MB-231 cell surface expression of CD73 by CD73 siRNA transfection and observed only 20–30% expression of normal levels (Supplemental Fig. 2B). Incubation of these cells with AD2 mAb followed by tail vein injection into immunodeficient mice ($n = 6$ in each group) continued to inhibit metastasis formation in the lungs compared with controls, indicating that AD2 mAb treatment inhibits metastasis formation even for cancer cells with significantly reduced CD73 expression levels (Fig. 4F, 4G).

The growth rate of metastases already established in the lungs was equal in the two treated groups (AD2 mAb versus control mAb) in both the spontaneous and tail vein metastasis models, indicating that the Ab affected the establishment of the cancer cell's ability to extravasate and/or colonize the lungs, but not the proliferation rate

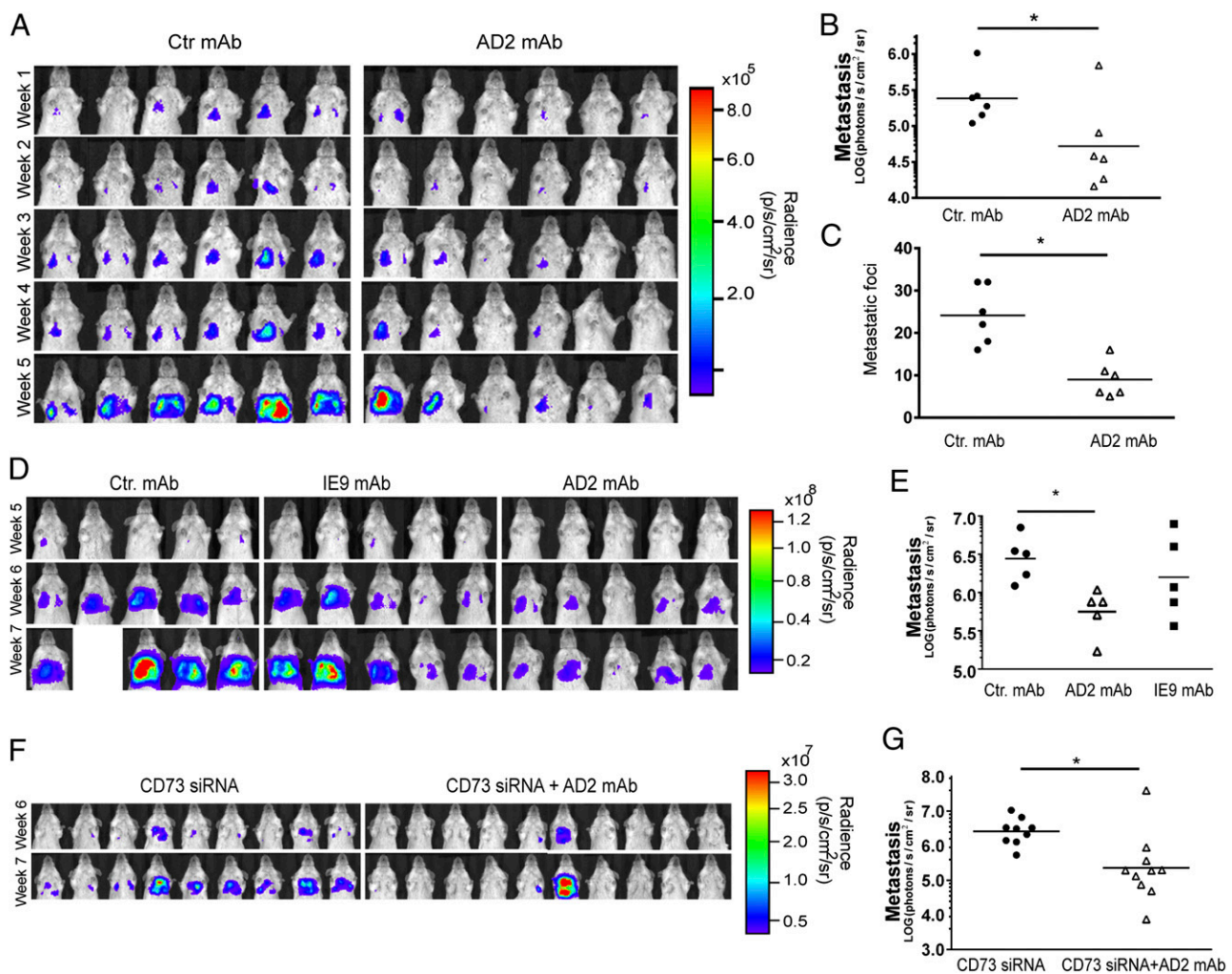


FIGURE 4. Ex vivo incubation of MDA-MB-231 cells with anti-CD73 AD2 mAb, but not anti-CD73 IE9 mAb, inhibits metastasis formation after i.v. injection. MDA-MB-231 cells (1×10^6) were incubated ex vivo with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb for 2 h and subsequently injected into the tail vein of female CB-17 SCID mice. **(A)** Lung metastasis development in mice, injected with MDA-MB-231 cells incubated ex vivo with either anti-CD73 AD2 mAb or control mAb, was monitored by weekly bioluminescence imaging (each row depicts the 2×6 animals at a given week, 1–5 wk). **(B)** The relative photon radiance per area from the pulmonary region in live animals ($n = 6$ per group) at week 6 was compared using a Student t test ($*p < 0.05$). **(C)** Furthermore, the metastasis development was evaluated by comparing the number of metastatic foci by immunohistochemistry ($n = 6$ per group) at week 6 using the Student t test ($*p < 0.001$). **(D)** In a second experiment, metastasis development in mice injected with MDA-MB-231 cells incubated ex vivo with either anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb was monitored by bioluminescence imaging. **(E)** The relative photon radiance per area from the pulmonary region of live animals ($n = 5$ per group) at week 6 was compared using Student t test (anti-CD73 AD2 mAb versus control mAb: $*p < 0.05$, anti-CD73 IE9 mAb versus control mAb: $*p > 0.05$). **(F)** The effect of AD2 mAb on metastasis development in animals injected with MDA-MB-231 cells that reduced CD73 levels (transfected with CD73 siRNA) incubated ex vivo was evaluated using bioluminescence. **(G)** The relative photon radiance per area from the pulmonary region of live animals ($n = 10$ per group) at week 7 was compared using the Student t test ($*p < 0.05$).

($p > 0.05$, random-effects model statistics). A critical step in cancer cell survival and metastasis after entry into the circulation is their ability to establish anchor-independent growth. However, cancer cells treated with AD2 mAb showed no impaired ability of anchor-independent growth compared with cancer cells treated with control mAb, as determined by a soft-agar anchor-independent growth assay (Supplemental Fig. 2C).

Anti-CD73 AD2 mAb has negligible influence on the catalytic activity of CD73

Treatment of MDA-MB-231 and LM3 cells with AD2 mAb (up to 70 mg/ml) reduced the catalytic activity of CD73 in vitro by only 18 ± 4.35 (SD) and $49 \pm 1.81\%$, respectively, compared with control mAb-treated cells (Fig. 5A, 5B). In contrast, the selective CD73 inhibitor APCP promoted a 93 ± 1.5 and $96 \pm 3.14\%$ inhibition of the catalytic activity of CD73 in MDA-MB-231 and LM3 cells, respectively (Fig. 5A, 5B).

To reiterate the setup of the tail vein injection experiments, we measured the catalytic activity of CD73 in MDA-MB-231 and LM3 cells when excess Ab was removed after preincubation of the cancer cells with AD2 mAb and IE9 mAb in vitro for 2 h at 37°C . The catalytic activity after AD2 mAb and IE9 mAb treatment decreased 11 ± 6.64 and $40 \pm 18.9\%$, respectively, in MDA-MB-231 (Fig. 5C). Preincubation of LM3 cells with AD2 mAb demonstrated a $52 \pm 3.0\%$ inhibition of CD73 catalytic activity, whereas preincubation of LM3 cells with IE9 mAb resulted in a remarkable $91 \pm 4.85\%$ inhibition of CD73 catalytic activity (Fig. 5D). Interestingly, CD73 activity in LM3 cells after AD2 mAb treatment increased $\sim 20\%$ only 3 h after removal of excessive Ab, demonstrating a short-lived effect of anti-CD73 mAbs, indicating the effect on the catalytic activity is functionally unimportant in an in vivo setting where a longer lasting effect is preferred (Fig. 5D). It should be noted that the effect of APCP was lost after washing the cells (Fig. 5C, 5D). Interestingly, upon knockdown of CD73 to $\sim 50\%$ in the highly expressing MDA-MB-231 cell line, no reduction in catalytic activity was observed compared with untransfected cells (Fig. 5E).

Anti-CD73 AD2 mAb and Fab' fragments of AD2 mAb, but not anti-CD73 IE9 mAb, induce alterations of CD73 distribution in the plasma membrane

The distribution of CD73 in the plasma membrane upon AD2 mAb incubation was investigated using live cell confocal immunofluorescence microscopy. AD2 mAb incubation of MDA-MB-231 cells at 37°C induced clustering of CD73 in the membrane and subsequent internalization of CD73 commencing ~ 10 min after incubation began (Fig. 6A, Supplemental Video 1). In contrast, the IE9 mAb induced minimal, if any, clustering and internalization of CD73 in MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is epitope dependent (Fig. 6B). Interestingly, Fab' fragments of the AD2 mAb also induced clustering and internalization of CD73 upon incubation of MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is not dependent on cross-linking of CD73 (Fig. 6B).

Internalization of anti-CD73 mAbs was further investigated using flow cytometry of live cancer cells. Analysis of CD73 expression after AD2 mAb incubation at 37°C and a subsequent round of AD2 incubation at 4°C showed that the mean fluorescence intensity (MFI) of CD73 staining decreased in MDA-MB-231 and LM3 cells compared with cells incubated at 4°C , whereas no decrease in CD73 MFI was observed after IE9 mAb incubation of MDA-MB-231 cells (Supplemental Fig. 3A). An increased MFI was observed after incubation of LM3 cells with IE9 mAb at 37°C compared with 4°C , which further supports that IE9 does not induce

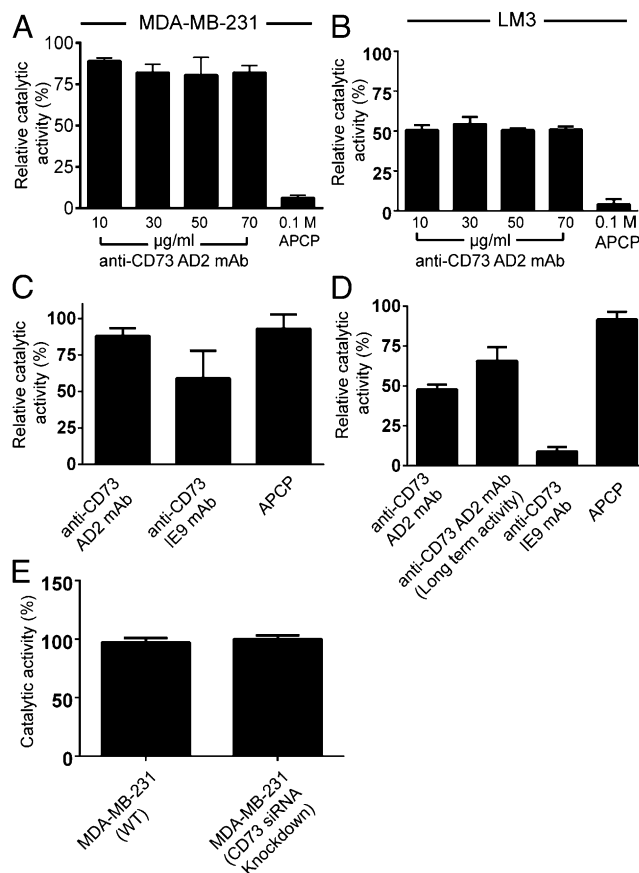


FIGURE 5. Anti-human CD73 mAb has a negligible influence on CD73 catalytic activity compared with APCP. The catalytic activity of CD73 in cancer cells was determined by measuring the generated inorganic phosphate from the enzymatic cleavage of AMP. (A) The catalytic activity in MDA-MB-231 cells treated with anti-CD73 AD2 mAb or APCP for 3 h relative to untreated control cells. (B) The catalytic activity in LM3 cells treated with anti-CD73 AD2 mAb or APCP for 3 h relative to untreated control cells. (C) The catalytic activity in MDA-MB-231 cells after pre-treatment with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or APCP followed by washing, thus resembling the in vivo tail vein injection experiments relative to untreated control cells. (D) The catalytic activity in LM3 cells after pretreatment with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or APCP followed by washing, thus resembling the in vivo tail vein injection experiments relative to untreated control cells. The anti-CD73 AD2 mAb (long-term activity) bar describes the relative catalytic activity in LM3 cells incubated for 2 h, washed, and incubated in full medium for 3 h without anti-CD73 AD2 mAb as compared with untreated control cells. (E) Catalytic activity in MDA-MB-231 cells after siRNA knockdown of CD73 and untransfected cells. CD73 expression after siRNA knockdown was $\sim 50\%$.

internalization of CD73 (Supplemental Fig. 3A). Analysis of CD73 expression in MDA-MB-231 omitting the second round of AD2 mAb incubation at 4°C showed a slight decrease in MFI compared with those incubated with two rounds of AD2, suggesting a minor re-expression of CD73 during the incubation period at 37°C (Supplemental Fig. 3B). This decrease in MFI was not observed in LM3 cells (Supplemental Fig. 3B). Furthermore, analysis of CD73 expression after AD2 incubation at 37°C and subsequent incubation in Ab-free medium showed that only $\sim 10\%$ of CD73 was re-expressed after 3 h (Supplemental Fig. 3B).

Discussion

CD73 has recently been proposed as a target for anticancer therapy (8, 9, 11), because treatment of mice with anti-murine CD73 mAb affects tumor progression by inhibiting the CD73-mediated pro-

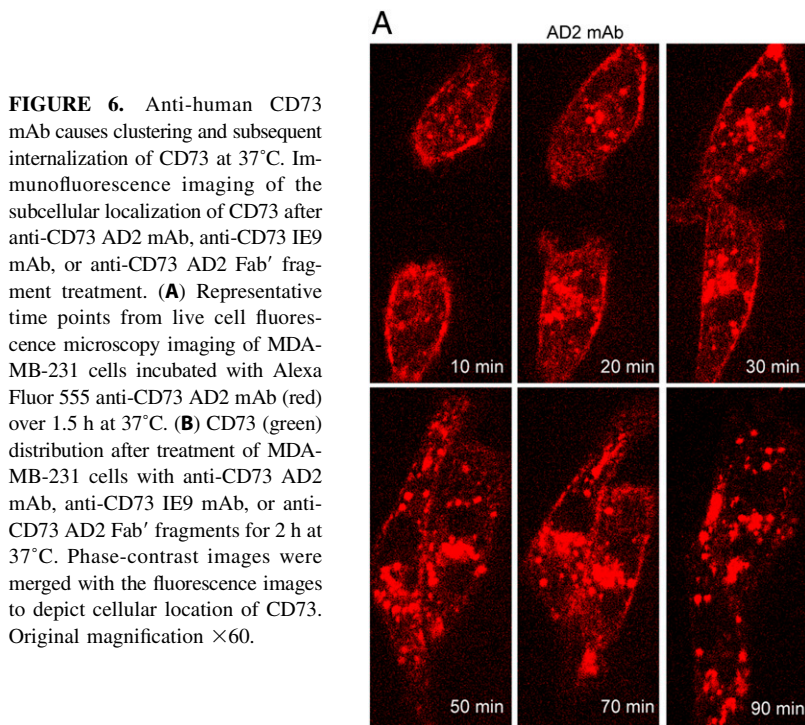


FIGURE 6. Anti-human CD73 mAb causes clustering and subsequent internalization of CD73 at 37°C. Immunofluorescence imaging of the subcellular localization of CD73 after anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or anti-CD73 AD2 Fab' fragment treatment. **(A)** Representative time points from live cell fluorescence microscopy imaging of MDA-MB-231 cells incubated with Alexa Fluor 555 anti-CD73 AD2 mAb (red) over 1.5 h at 37°C. **(B)** CD73 (green) distribution after treatment of MDA-MB-231 cells with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or anti-CD73 AD2 Fab' fragments for 2 h at 37°C. Phase-contrast images were merged with the fluorescence images to depict cellular location of CD73. Original magnification $\times 60$.

duction of adenosine. In this study, we provide novel insight to the role of CD73 in metastasis development and a novel mechanism by which some anti-CD73 Abs can inhibit metastasis formation independent of the catalytic activity of CD73. Our study indicates that CD73 redistribution, induced by anti-CD73 AD2 mAb (AD2 mAb), is central in the inhibition of human breast cancer metastasis in the lungs. Our data support the growing evidence suggesting CD73 as an attractive candidate for targeted treatment in breast cancer, and we suggest using the dual role of CD73 in metastasis development to increase the efficacy of anti-human CD73 mAb treatment by targeting the different functions of CD73.

The functions of CD73 can be divided into those relating to its catalytic activity, converting AMP into adenosine, and those independent of such activity, for example, cell adhesion and signal transduction (10). Most studies on CD73, including those in cancer, have focused on the functions related to the catalytic activity showing that adenosine has a broad range of effects on tumor progression, including promoting angiogenesis, immunosuppression, and metastasis development (7–9, 11, 16). It was recently shown that CD73 on cancer cells promotes spontaneous metastasis by enhancing cancer-cell migration in an adenosinergic manner through the adenosine A2B receptor and downstream promigratory mechanisms (8). Our *in vivo* data confirmed a role for CD73 in spontaneous metastasis of tumors established from the human breast cancer line LM3, and although the AD2 mAb exhibited a moderate effect on adenosine production in the LM3 breast cancer cells, this was shown to be short-lived and, therefore, functionally negligible. Furthermore, we show that AD2 mAb treatment inhibits metastasis after tail vein injection of MDA-MB-231 cells, but has only a slight effect on the catalytic activity *in vitro*. Moreover, when 80% of adenosine generating CD73 was removed by siRNA knockdown in MDA-MB-231 cells, AD2 mAb continued to significantly inhibit metastasis, further suggesting that the antimetastatic effect of AD2 mAb is independent of catalytic activity. Taken together, our data add yet another mechanism of CD73 in metastasis formation in human breast cancer in addition to the catalytic activity. Moreover, the inhibitory effect of AD2 mAb on

metastasis development was significant in the CD73-expressing LM3 and MDA-MB-231 cells, as well as the CD73 siRNA-treated MDA-MB-231 cells with reduced level of CD73, suggesting that the anti-metastatic effect of AD2 mAb is independent of CD73 expression levels.

Our study showed that AD2 mAb inhibited metastasis formation whereas having no effect on the growth of established primary tumors or metastasis. Furthermore, we showed that *ex vivo* preincubation of human cancer cells with AD2 mAb inhibited metastasis after tail vein injection, which circumvents the early steps of the metastatic process, suggesting that CD73 promotes an essential function in extravasation, colonization, or both. Together, this indicates that AD2 mAb inhibits metastasis formation independent of an effect on the primary tumor. This suggestion is supported by our previous study with isogenic cancer cell lines wherein we identified CD73 as one of the markers overexpressed in the cancer cell line that was capable of colonization in distant organs compared with its isogenic partner: a cancer cell line that could spread from the primary tumor but remained dormant in the distant organ (17).

Incubation of the breast cancer cells with AD2 mAb and AD2 Fab' fragments, but not anti-CD73 IE9 mAb (IE9 mAb) *in vitro*, caused CD73 to cluster and subsequently internalize, indicating that the process is epitope specific, but does not depend on cross-linking of CD73. It is well recognized that although some cell-surface proteins are internalized only when cross-linked by one or two layers of Abs (primary and secondary Abs), other receptors are internalized by binding of Fab' fragments of an Ab (18–20). IE9 and AD2 mAbs are both generated from mice immunized with full-length native CD73, however, as we showed IE9 and AD2 mAbs recognized different epitopes about which little is known other than the fact that IE9 mAb, in contrast with AD2, recognizes denatured CD73, suggesting that IE9 recognizes a linear epitope of CD73, whereas AD2 mAb recognizes a conformational epitope.

Ex vivo preincubation of MDA-MB-231 tumor cells with IE9 did not significantly inhibit metastasis development compared with control mAb-treated tumor cells. Treatment of cancer cells with

AD2 mAb, but not IE9, might trigger alterations of CD73 distribution in the plasma membrane and internalization, disrupting prometastatic functions of CD73. The internalization of CD73 might occur concomitantly as a result of CD73 redistribution, and the lower CD73 expression resulting from internalization might not cause inhibition of metastasis development per se. Although the fluorescent microscopy and the flow cytometry data of AD2 mAb supported each other, the readouts were not directly proportional, likely because of technical rather than biological reasons. Re-expression of CD73 in MDA-MB-231 cells after 3-h incubation with AD2 mAb at 37°C were shown to be only ~10%. A recent study suggested that CD73, upon ligand binding, might interact with other membrane proteins and cytoskeleton components to promote cell-stroma adhesion (21). AD2-induced reorganization of CD73 on the surface of the tumor cells, sustained for a substantial period of time, might prevent metastasis development through interference with these proposed intermembrane interactions. Although inducing internalization of CD73 AD2 had no effect on the enzymatic activity, knockdown of CD73 to ~50% in the highly expressing MDA-MB-231 cell line resulted in no reduction in catalytic activity compared with untransfected cells. These data indicate that, in cells expressing high levels of CD73, these levels are not directly proportional to the enzymatic activity.

Based on data showing CD73 clustering and internalization, we propose that CD73, independent of its function as an adenosine-generating enzyme, acts as an adhesion and/or signaling molecule. This is also supported by the study of Zhi et al. (12) showing that knockdown of CD73 in human metastatic MDA-MB-231 cells more efficiently prevented adhesion to the extracellular matrix than APCP treatment. This study did not demonstrate the impact of siRNA knockdown of CD73 on metastasis development; however, because our data indicate the effects of AD2 mAb treatment are independent of CD73 expression levels, it would be interesting to investigate the direct correlation between CD73 expression and metastasis development. Dual roles of other GPI-anchored CD proteins have previously been demonstrated. CD157, which exhibits ADP-ribosyl cyclase and cyclic ADP ribose hydrolase activities, has been shown to be important for neutrophil migration and adhesion independent of the catalytic activity of CD157 (22).

Recent studies wherein cancer cells were injected into the tail vein of syngeneic mouse models with various cancer types showed that treatment with APCP or anti-murine CD73 mAb before cancer cell injection inhibited lung metastasis independent of T and NK cells, suggesting that CD73 expressed by other host cells, for example, endothelial cells, also plays an important role in the ability of circulating tumor cells to metastasize (11, 23). Because AD2 mAb recognizes only human and not murine CD73, we conclude that the metastasis formation and inhibition thereof observed in our animal models is dependent solely on the CD73 expressed on the human cancer cells. It should be noted, however, that in humans, we cannot exclude a metastasis-promoting function of CD73 of normal adjacent cells, including endothelial cells, present in the metastatic niche. These cells should also be blocked by systemic treatment with anti-human CD73 mAb.

Studies in syngenic breast cancer mouse models have shown that the tumor-promoting effect of CD73 was mediated through the enzymatic function of CD73 by inhibiting the T cell-mediated antitumor response (8, 9, 11). Because our animal studies were performed in xenograft models using immunocompromised animals, the effect of CD73 on the immune system could be ruled nonimportant in our model. However, an Ab designed to inhibit catalytic activity of CD73 in human breast cancer would probably, in the presence of a functional immune system, favor an antitumor

immune response leading to inhibition of primary tumor and metastasis growth.

In this study, we show that an anti-human CD73 mAb reduces the ability of circulating human cancer cells to establish secondary tumor foci by inducing CD73 clustering and internalization. The metastatic effect of CD73 seems independent of its catalytic function of generating adenosine, but is instead attributable to the adhesive and/or signaling properties of CD73. Our data suggest that a mixture of anti-human CD73 Abs targeting different functions of CD73, such as adhesion and catalytic activity, would be therapeutically beneficial and also reduce the risk for developing resistance toward the anti-CD73 mAb treatment.

Acknowledgments

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Disclosures

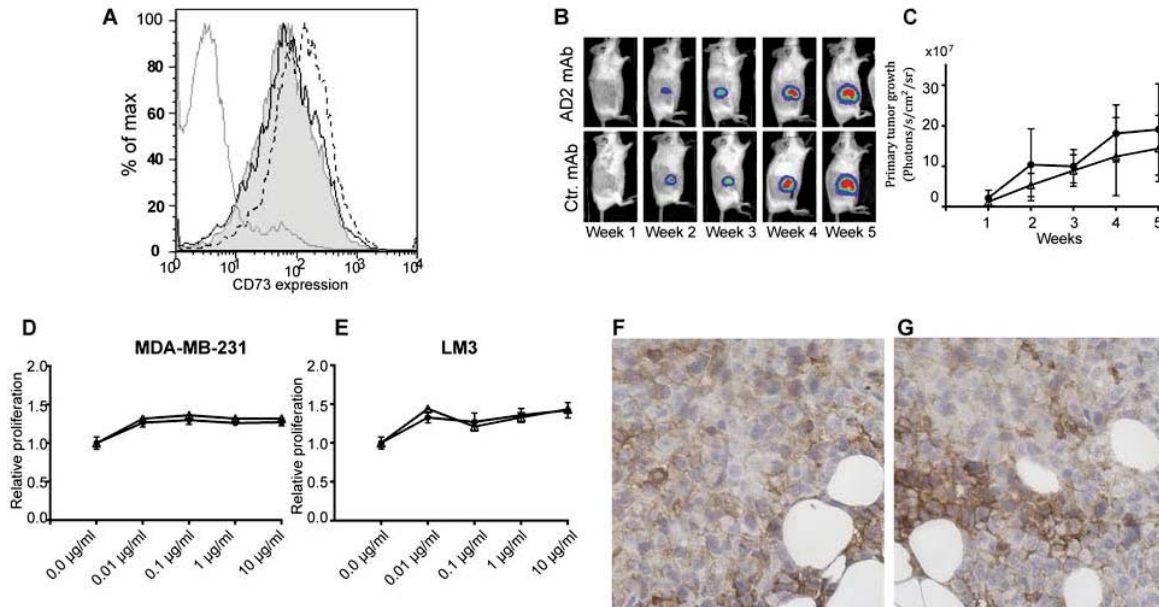
The authors have no financial conflicts of interest.

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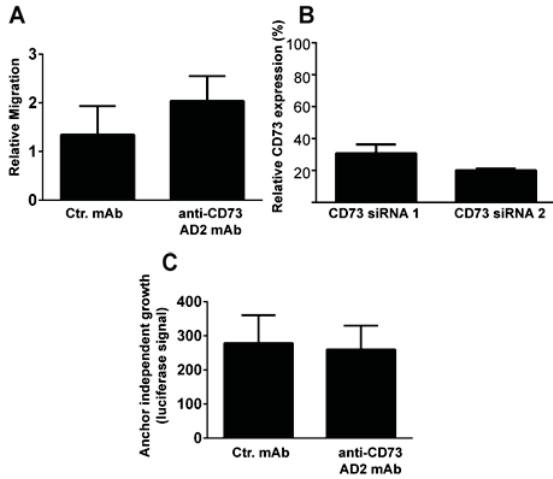
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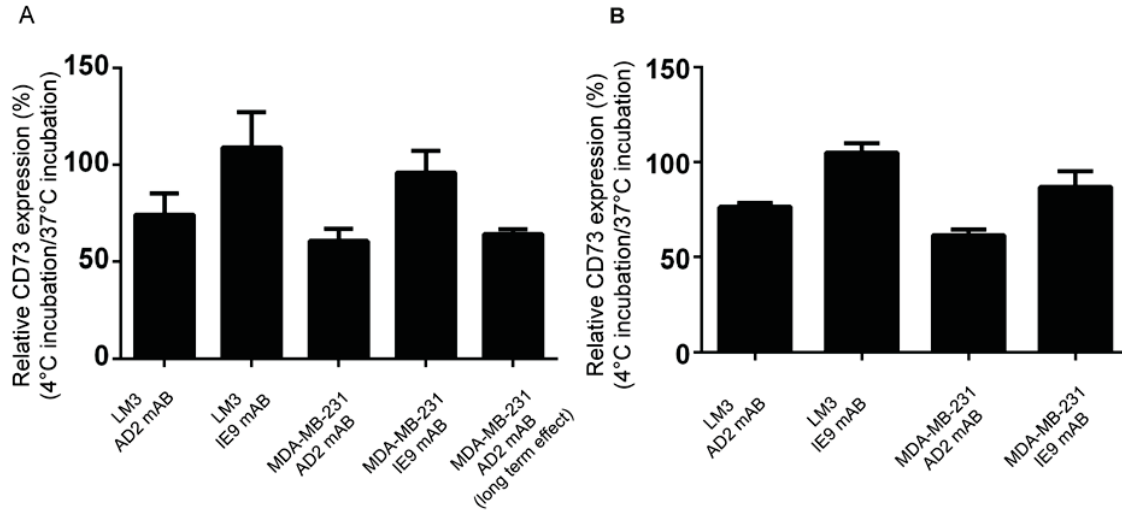
SUPPLEMENTARY DATA



Supplemental Figure 1. (A) Flow cytometry analysis of anti-CD73 AD2 mAb (black line), anti-CD73 IE9 mAb (solid gray), combination of anti-CD73 AD2 mAb and anti-CD73 IE9 mAb (tinted black line) and control mAb (gray line) in LM3 cancer cells. (B) LM3-2 cells (10^6) were transplanted into the mammary fat pad of female CB-17 mice and anti-CD73 AD2 mAb (5 mg/kg) or control mAb (5mg/kg) was administered at day 4 and subsequently twice a week for 5 weeks. A representative mouse from each group was imaged weekly for luciferase activity using the IVIS Spectrum instrument. (C) Comparison of photon radiance per area between the two groups of mice at week 5 using a Student's t-test ($p > 0.05$). (D) MDA-MB-231 and (E) LM3 cells were grown for 96 h after incubation with anti-CD73 AD2 mAb or control mAb and proliferation was subsequently measured using the MTT assay. (F-G) Immunohistochemical analysis of complement activation factor C3d in primary tumors from mice treated with (F) anti-CD73 AD2 mAb or (G) control mAb.



Supplemental Figure 2. Anti-CD73 AD2 mAb does not inhibit migration or anchor independent growth of cancer cells *in vitro*. (A) Migration of MDA-MB-231 cells treated with either anti-CD73 mAb or control mAb in a premade scratch after 48h was evaluated by comparing the quantification of segmented darkfield images of migrating cells using Image J. Each scratch was normalized to the quantification of the starting point 3h post-scraping. (B) CD73 expression in MDA-MB-231 cells 96h after siRNA transfection with two different CD73-specific siRNAs relative to CD73 expression in control scrambled siRNA transfected cells. (C) MDA-MB-231 cells were incubated in the mid layer of three semisolified soft agar layers supplemented with growth medium and either anti-CD73 AD2 mAb or control mAb for 12 days. Anchored independent growth was quantified by measuring bioluminescence using a Victor 3 Multilabel Plate Reader (PerkinElmer Life Science).



Supplemental Figure 3. Anti-CD73 AD2 mAb, but not anti-CD73 IE9 mAb, decreases cell surface expression of CD73 at 37°C compared to incubation at 4°C. (A) CD73 surface expression in MDA-MB-231 and LM3 was determined by flow cytometry following incubation of tumor cells with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb at 37°C and a second round of primary antibody incubation at 4°C as shown by mean fluorescence intensity of CD73 signal relative to incubation at 4°C. The MDA-MB-231 AD2 mAb (long term effect) bar describes the relative expression in MDA-MB-231 cells following AD2 incubation at 37°C, wash, incubation in full medium for 3 hours without anti-CD73 AD2 mAb at 37°C and a second round of AD2 at 4 °C compared to incubation at 4°C. (B) CD73 surface expression in MDA-MB-231 and LM3 was determined by flow cytometry following incubation of tumor cells with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb at 37°C omitting the second round of primary antibody incubation as shown by mean fluorescence intensity of CD73 signal relative to incubation at 4°C.

Video Legend. Anti-human CD73 mAb causes clustering and subsequent internalization of CD73 at 37°C. Live cell fluorescence microscopy imaging of MDA-MB-231 cells incubated with AlexaFluor555 anti-human CD73 mAb (red) at 37°C. Clustering and internalization of CD73 in a representative time frame of 15 min starting 10 min after the antibody was added can be seen.